IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Schubert et al.

Serial No.: 09/372,036
Filed June, 06, 1993
For: PROCESS AND AGENTS FOR DETECTING LISTERIAS

Group Art Unit: 1645
Examiner: Baskar, P.

DECLARATION

Honorable Commissioner of
Patents and Trademarks
Washington, D.C., 200231

MADAM/SIR:

The Declarant, Andreas Bubert, being duly warned, declares and says:

THAT he is a German citizen, residing at 64401 Gross-Bieberau, Germany;

THAT he is a biologist having studied at the University of Würzburg, Germany, from 1981 to 1988;

THAT he graduated from the University of Würzburg in 1988,

THAT he obtained the Dr. rer. nat. degree in the field of microbiology and biochemistry from the University of Würzburg in 1993;

THAT he worked as a postdoctoral research associate at the University of Würzburg from 1993 to 1998;

Declaration by A. Bubert

In re application of Schubert et al.

Serial No.: 09/372,036

THAT, in 1998 he joined the Research and Development Department of the Scientific Laboratory Products (SLP) Division of MERCK KGaA, Darmstadt, Germany;

THAT, since 1992 he has been working in the fields of immunology and immunologic testing, including Listeria and Salmonella;

THAT he is author or co-author of numerous articles in the field of immunology and immunologic testing;

THAT he is inventor or co-inventor of numerous inventions in the field of immunology and immunologic testing;

THAT he is familiar with issues related to immunology and immunologic testing, especially with the subject invention disclosed and claimed in U.S. Patent Application Ser. No. 09/372,036, filed June 06, 1993, by P. Schubert et al (hereinafter referred to as Application), of which he is co-inventor;

THAT methods of screening antibodies and studying their specificity are conventional and known to persons skilled in the art;

THAT the experiments performed in Applied and Environmental Microbiology, Sept. 1994, p. 3120-3127 and in Appendix 1, clearly show that the antibodies generated according to the present Application do bind to p60 of *listeria monocytogenes*;

THAT wildtype *E. coli* does not produce p60;

Declaration by A. Bubert

In re application of Schubert et al.

Serial No.: 09/372,036

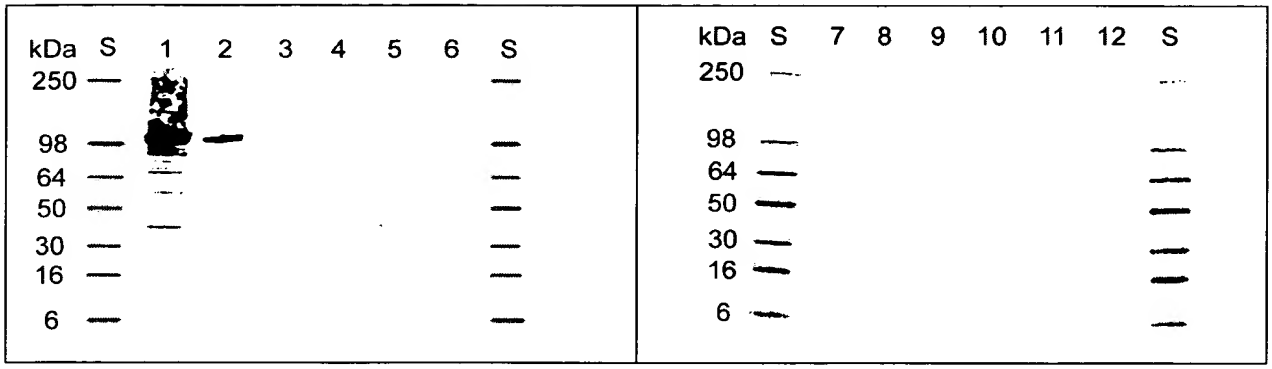
THAT in the experiment of Appendix 1 only those *E.coli* strains which were transformed with the pMALc2-p60-*monocytogenes* plasmids are able to produce a protein to which the antibodies according to the present application can bind. This shows that the antibodies according to the present application do bind to p60 and that they are specific for p60 of *Listeria monocytogenes*.

THAT the undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the APPLICATION or any patent issuing thereon.

Done, this May 07, 2001, at Darmstadt, Germany

Dr. Andreas Bubert

APPENDIX 1: Western Blot analyses of recombinant p60-producing *E. coli*-strains



S molecular weight standard + = induced by IPTG − = uninduced

1	*E. coli*/pMALc2-p60-*monocytogenes* +	7	*E. coli*/pMALc2-p60-*welshimeri* A +
2	*E. coli*/pMALc2-p60-*monocytogenes* −	8	*E. coli*/pMALc2-p60-*welshimeri* A −
3	*E. coli*/ pMALc2-p60-*innocua* +	9	*E. coli*/pMALc2-p60-*welshimeri* B +
4	*E. coli*/ pMALc2-p60-*innocua* −	10	*E. coli*/pMALc2-p60-*welshimeri* B −
5	*E. coli*/ pMALc2-p60-*ivanovii* +	11	*E. coli*/pMALc2 +
6	*E. coli*/ pMALc2-p60-*ivanovii* −	12	*E. coli*/pMALc2 −

Description:

Recombinant plasmids of pMALc2 were constructed that carry various types of *iap* genes from *Listeria*. The *iap* gene codes for p60 protein. These plasmids were used for transformation of *E. coli*.

All *E. coli* strains were enriched in culture media in which an inductor of protein expression (IPTG) was added for 4 hours. Then, cell lysate proteins containing recombinant (r)-p60 of recombinant *E. coli* strains were prepared and these were separated by SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes and incubated with a monoclonal antibody raised against the peptide QQQTAPKAPTE that had been derived from the *L. monocytogenes* p60 protein.

As shown in Lanes 3-12, the r-p60 protein of *L. monocytogenes* is neither produced by *E. coli* strains carrying only the plasmid pMALc2 alone nor by any *E. coli* strain that carries on pMALc2 a r-p60 of a species other than *Listeria monocytogenes*.

As shown in Lanes 2+3, only the *E. coli* strain that produces the r-p60 of *L. monocytogenes*, can be detected with this antibody. Since r-p60 is produced in *E. coli* as a fusion protein, the molecular weight is about 98 kDa. The additional bands that appear in lane 1 from E. coli pMALc2-p60-monocytogenes are degradation products of p60 which occur after overproduction (see Bubert et al., Applied and Environmental Microbiology, 1994, Vol. 60, p. 3120-3127).

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Peter SCHUBERT et al.

Serial No.: 08/412,227
Filed March 27, 1995
For: PROCESSES AND AGENTS FOR DETECTING LISTERIAS
Group Art Unit: 1806
Examiner: R. SCHWADRON

DECLARATION

Honorable Commissioner of
Patents and Trademarks
Washington, D.C., 200231

SIR:

The Declarant, Rolf Vormbrock, being duly warned, declares and says:

THAT he is a German citizen, residing at Darmstadt, Germany;

THAT he is a chemist having studied at the University of Münster, Germany, from 1968 to 1976;

THAT he graduated from the University of Münster, where he obtained the Dr. degree in the field of biochemistry in 1976;

THAT he worked as a postdoctoral research associate at the Technical University in Darmstadt from 1976 to 1977;

Declaration
In re application of Peter SCHUBERT et al.
Serial No.: 08/412,227
Filed March 27, 1995

THAT, in 1977 he joined the Research and Development Department of the Diagnostics Division of E. MERCK (now MERCK KGaA), Darmstadt, Germany;

THAT, since 1976 he has been working in the fields of biochemistry, clinical diagnostics, and clinical chemistry;

THAT he is author or co-author of numerous articles in the field of clinical chemistry;

THAT he is inventor or co-inventor of numerous inventions in the field of clinical chemistry;

THAT he is familiar with issues related to the diagnostic validity, e.g. diagnostic sensitivity and specificity, of diagnostic tests, and THAT such issues are presented in text books of Clinical Chemistry, e.g. in the text book by L.A. Kaplan and A.J. Pesce, C.V. Mosby (1989) (copies of pertinent pages enclosed);

THAT the issues related to diagnostic validity are an aspect of the invention disclosed and claimed in U.S. Patent Application Ser. No. 08/412,227, filed March 27, 1995, by Peter Schubert et al., which is a continuation of U.S. Patent Application Ser. No. 08/075,248, filed November 06, 1993 (hereinafter referred to as APPLICATION);

THAT the example given in the text book of L.A. Kaplan and A.J. Pesce on pages 267 - 269 can be applied to a situation, where the presence of pathogenic Listeria has to be tested; the equivalent categories are:

L.A. Kaplan and A.J. Pesce	APPLICATION
diseased	pathogenic Listeria present
not diseased	pathogenic Listeria absent
test positive	positive reaction
test negative	negative reaction

(Table A)

Declaration
In re application of Peter SCHUBERT et al.
Serial No.: 08/412,227
Filed March 27, 1995

consequently Table 17-3 of L.A. Kaplan and A.J. Pesce translates into:

	pathogenic Listeria present	pathogenic Listeria absent	Total
positive reaction	a	b	a + b
negative reaction	c	d	c + d
Total	a + c	b + d	N

sensitivity = a / (a + c)
specificity = d / (b + d)

(Table B)

THAT the following conclusions can be drawn, when the definitions outlined above are applied to the data presented in the Declaration by P. Schubert dated April 12, 1994 and March 22, 1995:

Date of Declaration	April 12, 1994			March 22, 1995
Experiment	APPENDIX B			APPENDIX
Peptide	Sequ.I.D. # 42	Sequ.I.D. # 30	total p60	Sequ.I.D. # 42
a	13	11	12	72
b	0	0	1	0
c	0	2	1 #	0
d	2	2	1	82
specificity	1.00	1.00	0.50	1.00
sensitivity	1.00	0.85	0.92	1.00

only marginal reaction
(Table C)

Declarati n
In re application of P ter SCHUBERT et al.
Serial No.: 08/412,227
Filed March 27, 1995

THAT the data of Table C show that tests for the detection of pathogenic Listeria based on antibodies raised by immunization of experimental animals with peptides of Sequ.I.D. No.'s 30 or 42 both show a specificity of 1.00; consequently these tests are specific for the detection of pathogenic Listeria;

THAT the undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the APPLICATION or any patent issuing thereon.

Done, this February 12, 1997 at Darmstadt, Germany

Rolf Vormbrock

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Peter SCHUBERT et al.

Serial No.: 08/075,248
Filed November 06, 1993
For: PROCESSES AND AGENTS FOR DETECTING LISTERIAS
Group Art Unit: 1806
Examiner: R. SCHWADRON

DECLARATION

Honorable Commissioner of
Patents and Trademarks
Washington, D.C., 200231

SIR:

The Declarant, Peter Schubert, being duly warned, declares and says:

THAT he is a German citizen, residing at Darmstadt, Germany;

THAT this DECLARATION is an addition to his earlier declaration dated April 12, 1994;

THAT the experiments outlined in the APPENDIX show results which could not be anticipated from the cited documents of prior art;

THAT the antibodies of the APPLICATION are highly useful for detecting Listeria monocytogenes;

./2

THAT these results are surprising in view of his earlier experience and not predictable by a person having ordinary skill in the art;

THAT the undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the APPLICATION or any patent issuing thereon.

Done, this March 22, 1995 at Darmstadt, Germany

Peter Schubert

DECLARATION in re application of
Peter SCHUBERT et al.
Serial No.: 08/075,248
Filed November 06, 1993
For: PROCESSES AND AGENTS FOR DETECTING LISTERIAS

APPENDIX

Experimental

1. Antibody used

The peptide according to Sequ.ID.No. 42, the immunogenic conjugate, and the antibody raised in rabbits were prepared as described in the earlier declaration (antibody against Peptide A in APPENDIX A). The peptide has the following sequence:

Cys Gln Gln Gln Thr Ala Pro Lys Ala Pro Thr Glu.

2. Strains

A total of 154 isolates and strains all belonging to the genus Listeria were tested. Biochemical typing using standard protocols was verified using PCR technology.

The strains tested included:

No	species	number of strains	including serovars:
1	L. innocua	31	1/2b; 6a; 6b
2	L. ivanovii	8	5
3	L. monocytogenes	72	1/2a; 1/2b; 1/2c; 3a; 3b; 4b; 4c
4	L. murrayi	2	
5	L. seeligeri	37	1/2a; 1/2b; 3b; 4b; 4c; 6b
6	L. welshimeri	4	6a; 6b

The number of isolates reflects the relative occurrences of different Listeria species in food samples.

3. Testing Procedure

Antibody against total p60 was adsorbed to microtiter plates using standard techniques.

1 ml of a sample culture containing at least 10^7 cells was heated for 10 minutes at 95 °C. The sample was allowed to cool to room temperature and was centrifuged for 10 minutes at 14,000 rpm (Eppendorf centrifuge Cat.No. 5415).

APPENDIX (continued)

100 µl clear supernatant from the previous step was pipetted into a well of a microtiter plate, incubated for one hour at 35 °C in a moisturized chamber, and washed three times.

100 µl of antibody solution (see 1. above) was pipetted into each well, incubated for one hour at 35 °C in a moisturized chamber, and washed three times.

100 µl of commercially available anti rabbit antibody conjugated with horseradish peroxidase was pipetted into each well, incubated for one hour at 35 °C in a moisturized chamber, and washed three times.

100 µl of substrate solution was pipetted into each well, incubated for 30 minutes at room temperature protected from light; after stopping the reaction the absorbance was measured at 410 nm.

Positive and negative controls were used to define the cut-off values.

4. Results

No	species	number of positive reactions	negative reactions
1	L. innocua	0	31
2	L. ivanovii	0	8
3	L. monocytogenes	72	0
4	L. murrayi	0	2
5	L. seeligeri	0	37
6	L. welshimeri	0	4

The data show that antibody raised against immunogenic peptides containing a peptide according to the present invention are useful to detect L. monocytogenes and to distinguish L. monocytogenes, which is a pathogen to humans, from other Listeria non-pathogenic to humans.